PharmaSat Optics Data Tutorial

This tutorial will introduce the AlamarBlue proliferation dye, explain how the yeast experiment data look and what they mean, introduce the PharmaSat reference standard filters, explain the reason for their selection, and illustrate how to interpret the data obtained from the optical reference standards contained in wells 24 - 35 of the PharmaSat experiment payload.

The fundamental nature of the PharmaSat experiment is to test the efficacy of various doses of antifungal drug in microgravity, as compared to their efficacy on the Earth's surface. The most critical measurement necessary to carry out this experiment is the growth rate of the organism (in this case yeast) before and after the administration of the dosage of antifungal drug. A common method of measuring this growth, or proliferation, is the use of a proliferation dye such as AlamarBlue.

AlamarBlue

The AlamarBlue assay employed in the PharmaSat experiment incorporates a colorimetric growth indicator based on detection of metabolic activity. The assay incorporates an oxidation-reduction (REDOX) indicator that changes color from dark blue to bright pink in response to chemical reduction of growth medium resulting from cell growth or proliferation. This specific proliferation dye has been carefully selected because of several properties:

- AlamarBlue (AB) exhibits colorimetric change in the appropriate oxidation-reduction range relating to cellular metabolic reduction.
- AB is demonstrated to be minimally toxic to living cells.
- AB produces a clear, stable, and distinct change, which is easy to interpret.

The AB assay is designed to quantitatively measure the proliferation of various human and animal cell lines, bacteria and fungi. The assay is simple to perform since the indicator is water soluble, thus eliminating the washing/fixing and extraction steps required in other commonly used cell proliferation assays. However, AB is only for use between pH6.8 and pH7.4, since pH levels beyond this range will effect a color change in the dye.

As the cells being tested begin to grow, innate metabolic activity results in a chemical reduction of AB. Continued growth maintains a reduced environment, while inhibition of growth maintains an oxidized environment. Reduction related to growth causes the REDOX indicator in AB to change from an oxidized (blue) form to a reduced (pink) form.

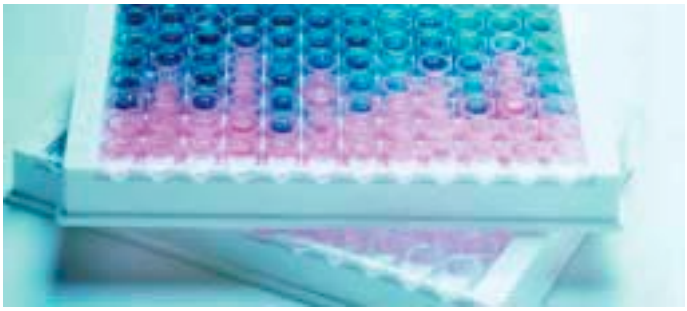


Figure 1 – Standard 96-well plate with AB treated cells demonstrating color change exhibited by growth (pink wells) and no-growth (blue wells)

Data are collected using absorbance-based instrumentation. Absorbance is typically monitored at 570nm and 600nm however in the PharmaSat optical system, absorbance (derived from transmittance) will be measured at 525nm (35nm half-width) and 615nm (18nm half-width).

The AB dye does not change from blue to pink in an instant. It occurs gradually as the chemical reduction of growth medium proceeds. When we look at the absorbance spectrum of visible wavelengths (400 nm – 700 nm), we can see visually how the dye changes in color over time. The color change itself is not an indicator of elapsed time, but an indicator of the percent reduction occurring in the sample, and typically takes place within a period of hours. In the case of an antifungal dosage that completely kills the sample, there will be no color change and the sample will remain blue.

Absorbance Spectra of AlamarBlue

As discussed above, the AlamarBlue assay incorporates an oxidation-reduction (REDOX) indicator that changes color from dark blue to bright pink in response to chemical reduction of growth medium resulting from cell growth or proliferation. The method of detecting this change in color is typically through absorbance measurements taken with a standard microwell plate reader. (PharmaSat will employ direct transmittance measurements taken with our custom optical system, which will be discussed further.)

The plot below illustrates absorbance measurements taken throughout a 10-hour period during which PharmaSat yeast samples were provided growth media and allowed to proliferate fully (no anti-fungal introduced). The absorbance spectra indicate how the absorbance peak shifts from longer wavelengths (~615 nm) to shorter wavelengths (~550 nm) during the growth phase. This shift in absorbance wavelengths is what enables us to measure the change in color in our payload environment, where neither human eyes nor imaging cameras are present.

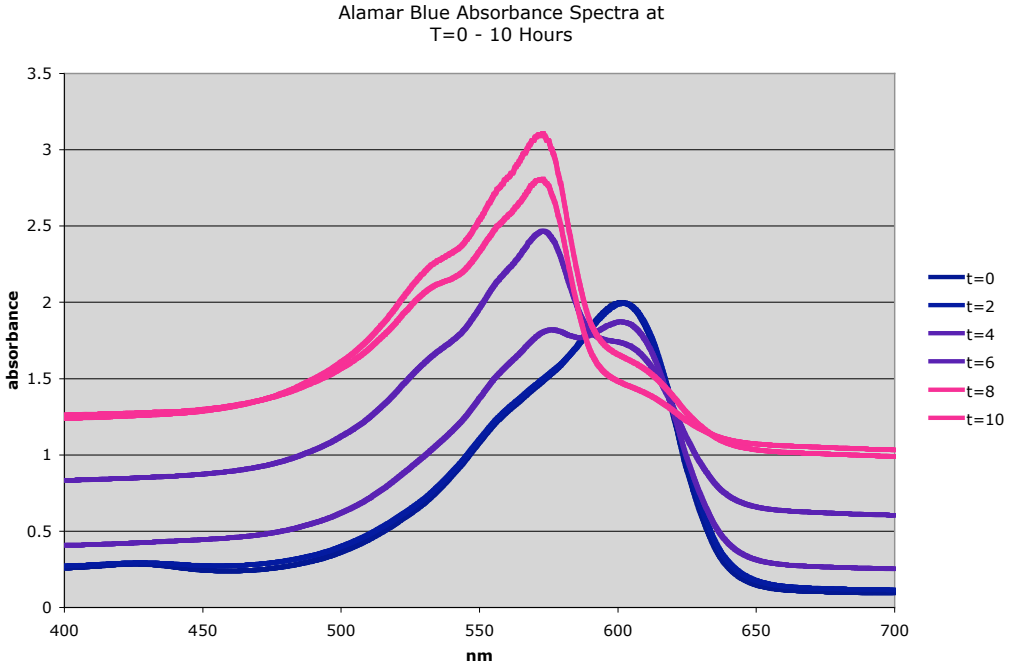
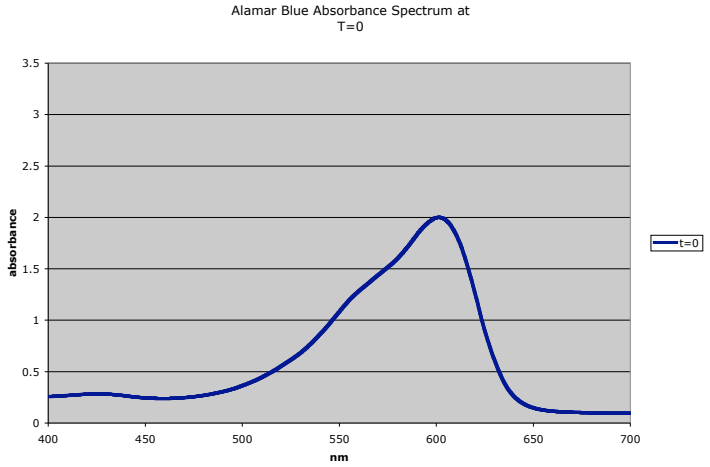
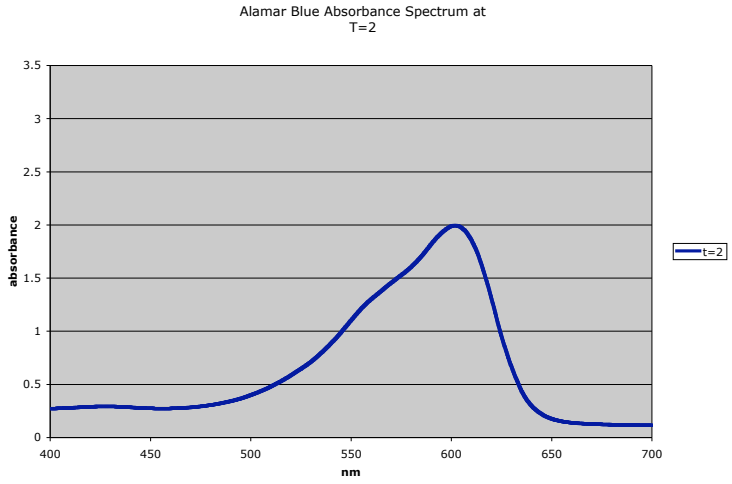
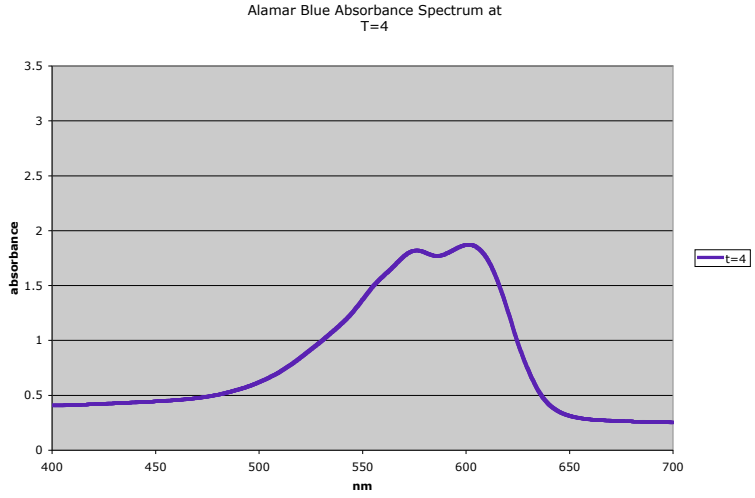


Figure 2 – Absorbance spectra of typical AlamarBlue-treated sample from oxidized (blue) to reduced (pink) form

Pictured on the following two pages are the individual absorbance spectra for each time point plotted above for (potentially) easier visualization of the shift in wavelength. The color coding of the time points correspond to the color expressed by AlamarBlue if you were observing the samples with your own eyes, or through an image-capture device.







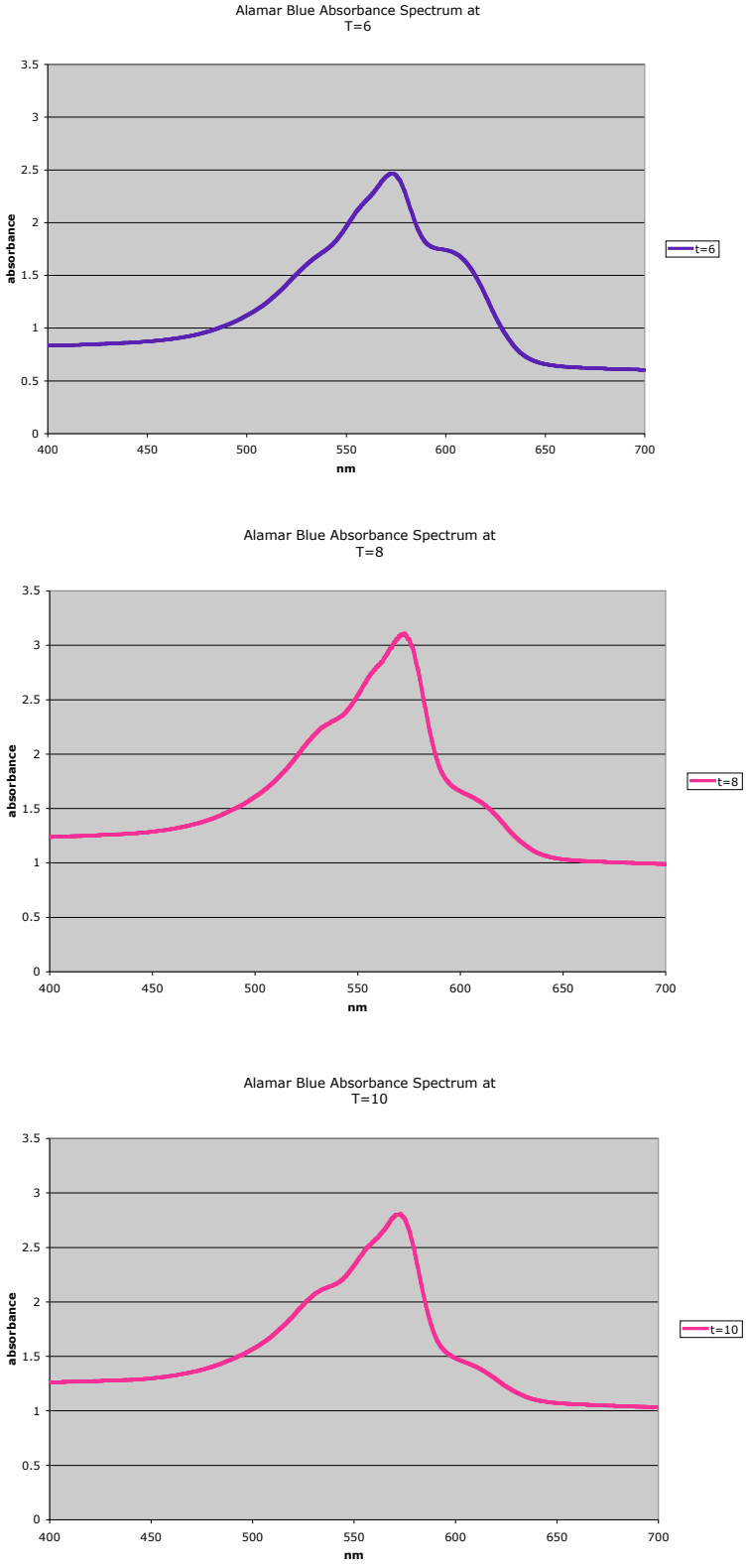


Figure 3 (pages 3 & 4) – Absorbance spectra of typical AlamarBlue-treated sample from oxidized (blue) to reduced (pink) form

If we then take each of these spectra and normalize them against the values obtained at the 470 nm wavelength (where minimal AB absorbance occurs), we can subtract out the optical density contribution to absorbance and visualize more easily the wavelength shift of interest resulting specifically from the reduction in AB dye.

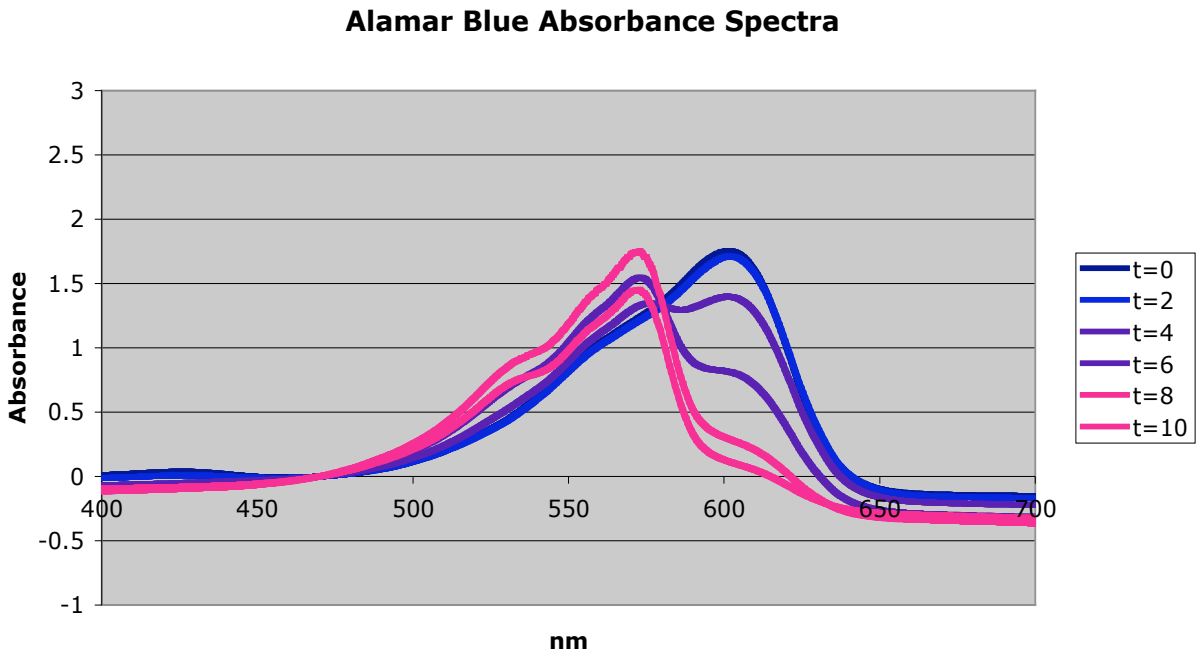


Figure 4 – Absorbance spectra of typical AlamarBlue-treated sample normalized to 470 nm

Absorbance Spectra of AlamarBlue with LED interrogation overlay

Adding an overlay of the LED wavelengths we use in the PharmaSat system for interrogation of the AB reduction demonstrates the color shift we expect to see with our detector, as shown in Figure 5 below. Specifically, when we interrogate with the red LED (peak wavelength 615nm, 18nm half-width) we see the signal drop significantly when comparing t=0 and t=10. Likewise, when we interrogate with the green LED (peak wavelength 525nm, 35nm half-width) we see the signal increase noticeably when comparing t=0 and t=10. Hence, if our yeast is in fact growing, we expect the absorbance measurement taken with the red LED to drop and the absorbance measurement taken with the green LED to rise. (In this assay it is only necessary to witness the signal change with the red LED; however, the green interrogation gives us a check that in fact our red signal did not drop for reasons other than AB reduction.)

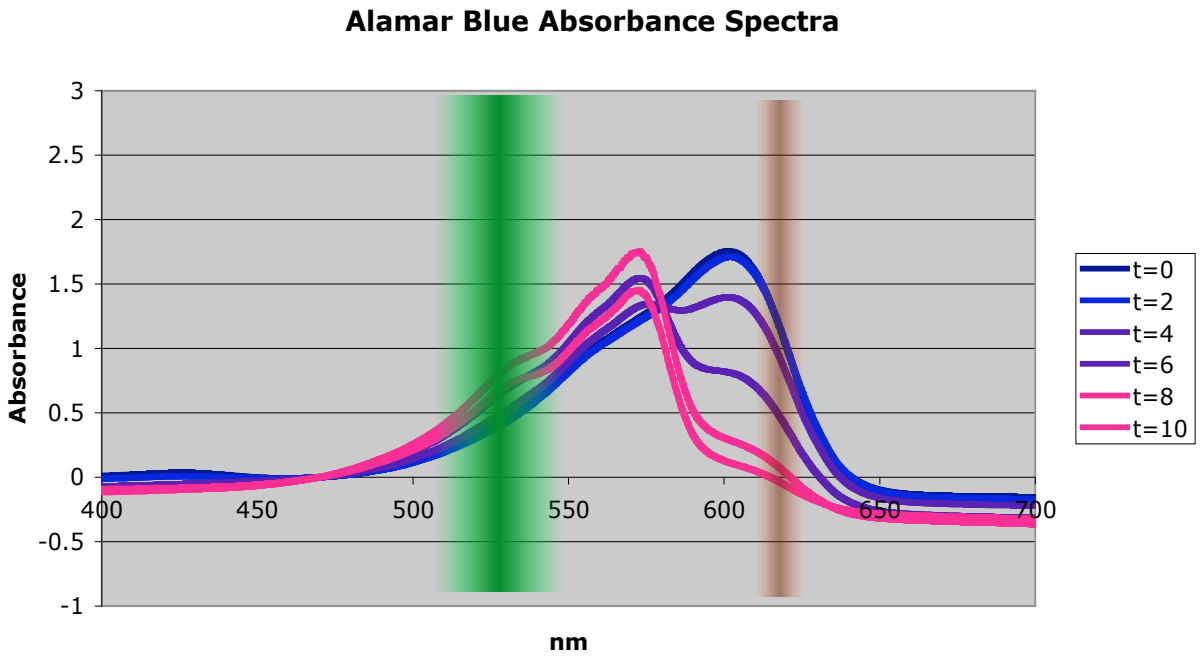


Figure 5 – Absorbance spectra of typical AlamarBlue-treated sample normalized to 470 nm, with overlay of PharmaSat interrogation wavelengths

Data Represented in PharmaSat Optical System

The data as we see them represented in the PharmaSat optical system look quite different. Below is a typical PharmaSat signal readout of a yeast sample treated with AB dye and allowed to fully proliferate. The first thing to notice is that three-color interrogation is employed as opposed to the two colors (red and green) discussed above. The additional blue LED is used to provide a measurement of optical density to assist in understanding the behavior of the yeast, independently of the AB proliferation test. This signal will be most useful in understanding at what phase of yeast growth the anti-fungal is introduced and for subtracting out the optical density contribution to the AB absorbance measurement (as was done on page 5 with the plate reader data).

The next obvious difference in the PharmaSat data is that the growth curves are nearly the *opposite* of the absorbance spectra we have been reviewing above. This is because the PharmaSat optics is taking transmittance measurements rather than absorbance. In other words, we are measuring the intensity of the light that actually passes through the yeast sample, as opposed to measuring the intensity of the light that is absorbed by the sample. Denoting by I_i and I_t the light intensities incident on and transmitted by the sample, respectively, the intensity transmittance of the sample is defined as $T = I_t/I_i$. The absorbance of the sample, in turn, is given by $A = -\log_{10}T$. In the PharmaSat system, an approximation will be utilized since our T will not be derived directly from $T = I_t/I_i$, but rather will be provided as a light intensity signal from our light-to-frequency detector which will be integrating a larger band of wavelengths than that measured by an absorbance plate reader.

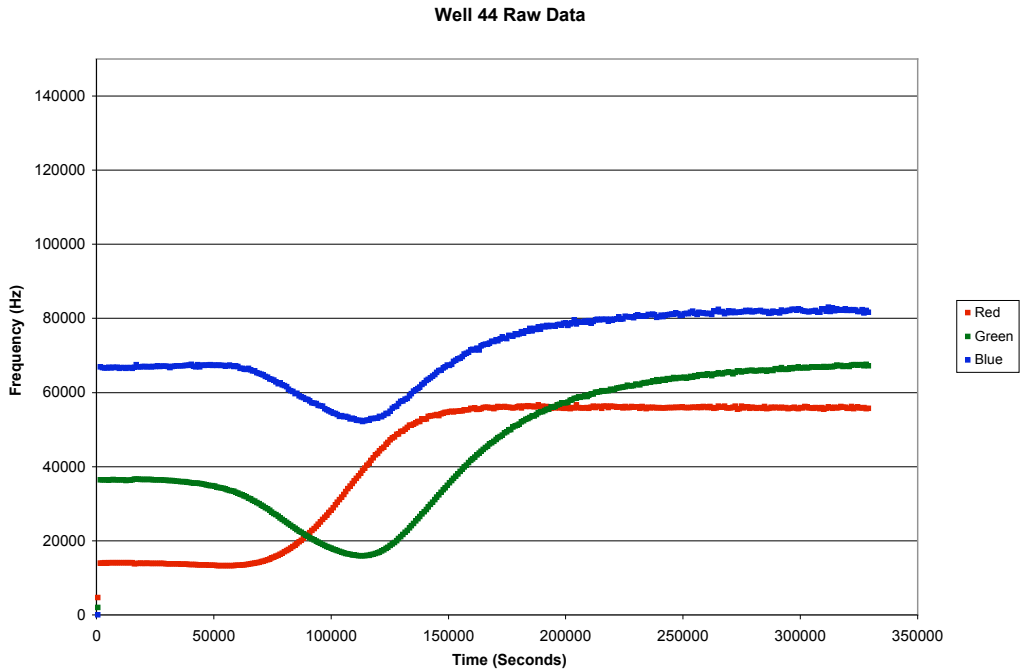


Figure 6 – Actual PharmaSat data from AB treated yeast, full growth curves

Therefore, when interpreting the raw PharmaSat data, the opposite rules apply to those used when interpreting absorbance. When the yeast sample is proliferating, we will see a steady increase in the red signal, accompanied by a drop (of a more gradual slope) in the green and blue signals, as is pictured above. The point when the signals begin to change direction is the end of the experiment.

At this stage, the AB REDOX indicator is breaking down and is no longer useful as a viability index. (In the plot above, data continued to be taken long after the experiment had completed.)

In the case of a yeast sample that does not proliferate (i.e., one where the antifungal dosage administered was effective in killing the sample), all three signals (red, green, blue) will remain essentially flat. In the case of a yeast sample that has been partially compromised (i.e., one where the antifungal dosage administered was effective in killing a portion of the sample, but did not inhibit the growth entirely), the three signals will behave as they do in a fully-proliferating sample (increase in red, decrease in green and blue), however the entire growth curve will be stretched out across the plot and there may be no intersections of the signals (very shallow slopes).

Layout of Reference Standards

The final topic of this tutorial is the reference standards incorporated into the payload. As illustrated in the fluidic card layout below, the reference line contains wells 24 – 35 with color filters (AB control) to the left of the card and neutral density filters (optical density control) to the right. The actual filters are placed inside the thermal spreader plate assembly where they are very securely mounted.

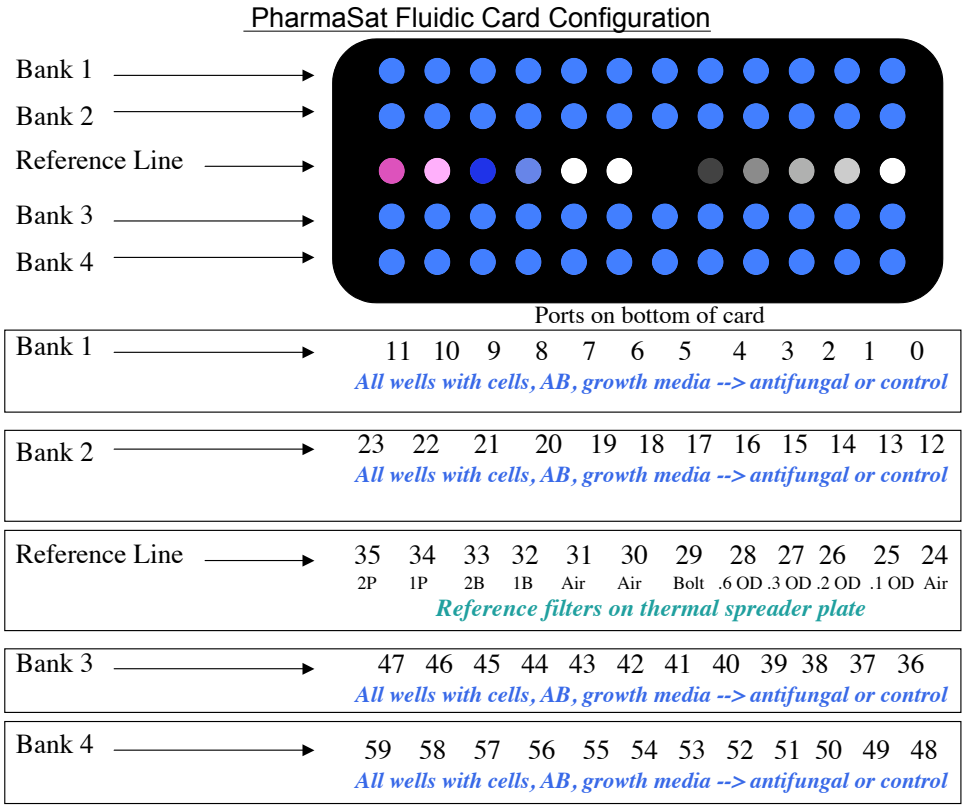


Figure 7 – PharmaSat fluidic card module layout (with reference standards placed inside thermal spreader plate assembly)

Neutral Density Filter (OD control) Reference Standards

The neutral density filters are placed in a linear fashion with increased light attenuation moving from the right to the left (wells 25 to 28). The actual light transmission of each filter is as follows:

Filter	% Light Transmission
.1 OD	79.4
.2 OD	63.0
.3 OD	50.1
.6 OD	25.1

When reviewing the data from these standard wells, the signal from all three LEDs should steadily decrease as the light transmission decreases. The four wells will not relate linearly to each other, as each well contains its own optical system with different baseline readings. However, a steady decrease in signal should be observable (samples provided on pages 11 - 13).

Color Filter (AB control) Reference Standards

The AB control color filters have been carefully chosen to represent the AB oxidized state (start of experiment) and AB reduced state (end of experiment). Their selection was based on similarity to actual AB absorbance spectra of t=0 and t=10 hours, as discussed in detail in earlier sections. Just as an actual AB sample in oxidized state (blue) will exhibit a stronger red signal than green, so will the AB oxidized reference standard (Figure 8). Similarly, just as an actual AB sample in reduced state (pink) will exhibit a stronger green signal than red, so will the AB reduced reference standard (Figure 9).

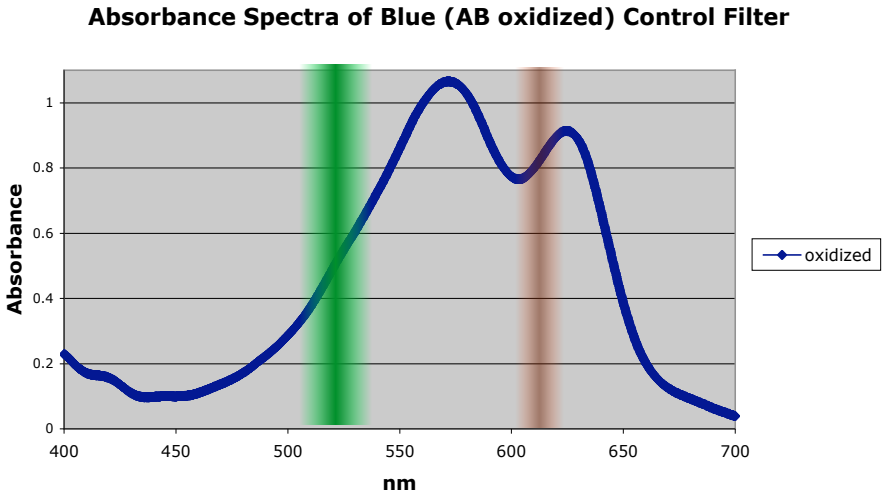


Figure 8 – Absorbance Spectra of Blue Color Filter (Control for AB Oxidized - Start of Experiment)

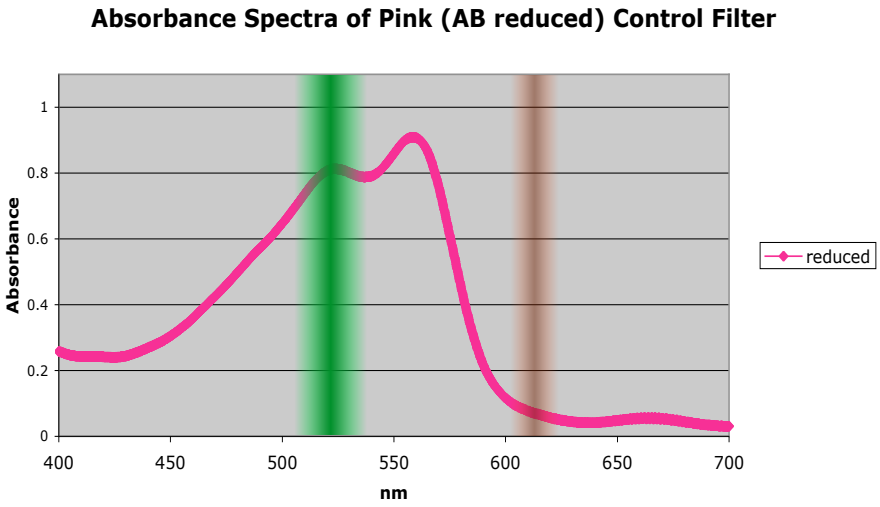
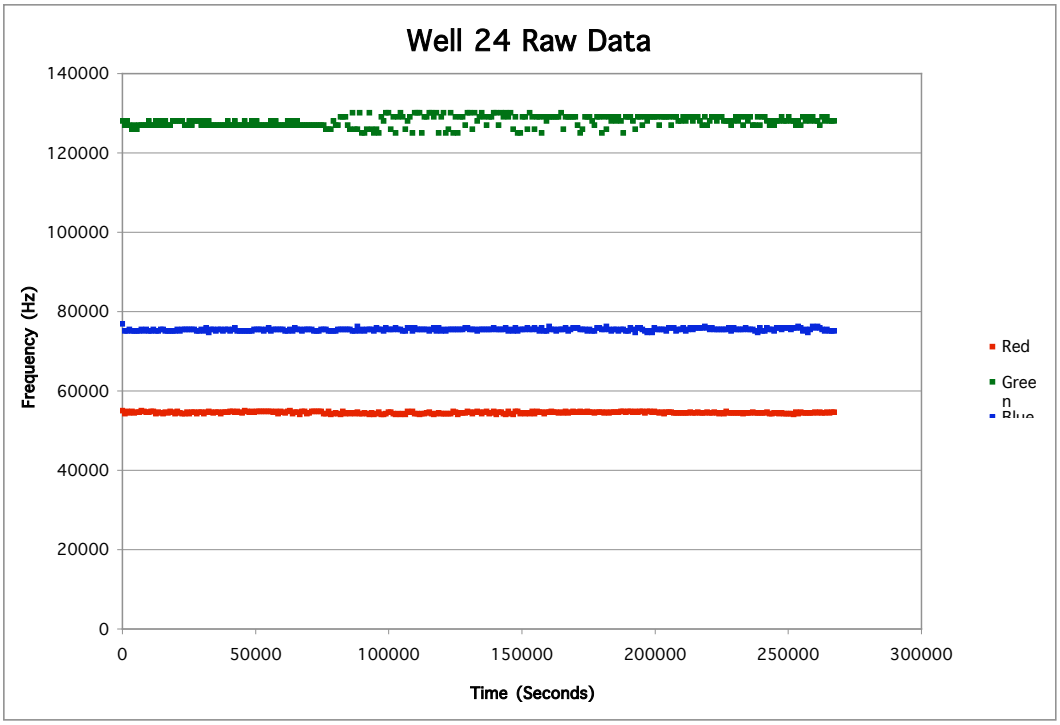


Figure 9 – Absorbance Spectra of Pink Color Filter (Control for AB Reduced - End of Experiment)

Sample Data from Reference Line

The data collected from the reference line wells should appear similar to the examples which follow on pages 11 - 16, taken from Optics Growth Test 2 (5/31/08). For actual flight units, PreSat or PharmaSat, we will have actual system checkout data taken pre-flight, which will provide the optics baseline for the reference line comparison. The key features to note are the following (summarized from filter sections above):

Well 24	Air	A measure of signal range with no light attenuation, no rules.
Wells 25 – 28	ND filters	Signal from all three colors should steadily decrease from well 25 to well 28.
Well 29	Bolt	No signal
Wells 30 – 31	Air	A measure of signal range with no light attenuation, no rules.
Well 32	Single Blue	Green signal should be much larger than red.
Well 33	Double Blue	Green signal should be much larger than red, with both intensities reduced when compared to Well 32.
Well 34	Single Pink	Red signal should be much larger than green.
Well 35	Double Pink	Red signal should be much larger than green, with both intensities reduced when compared to Well 34.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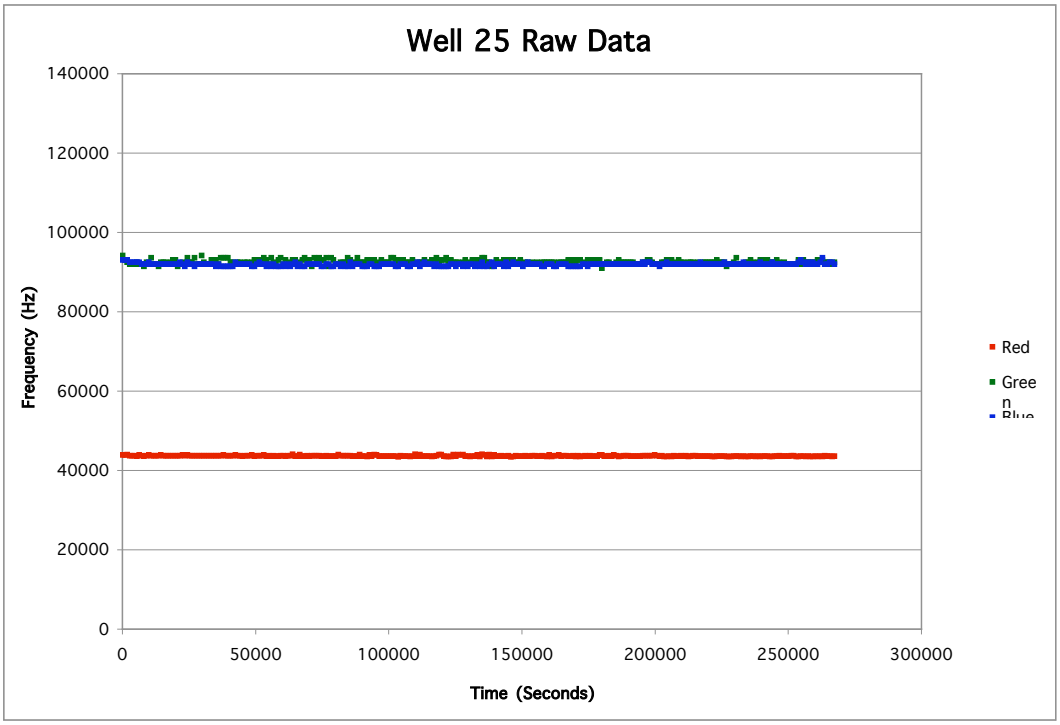
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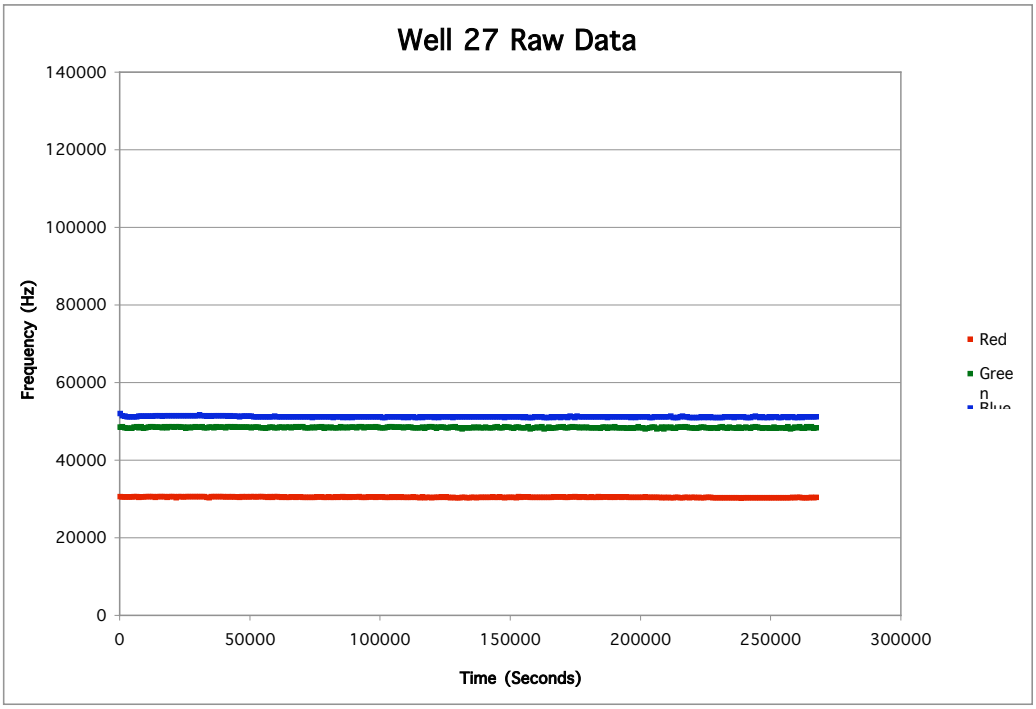
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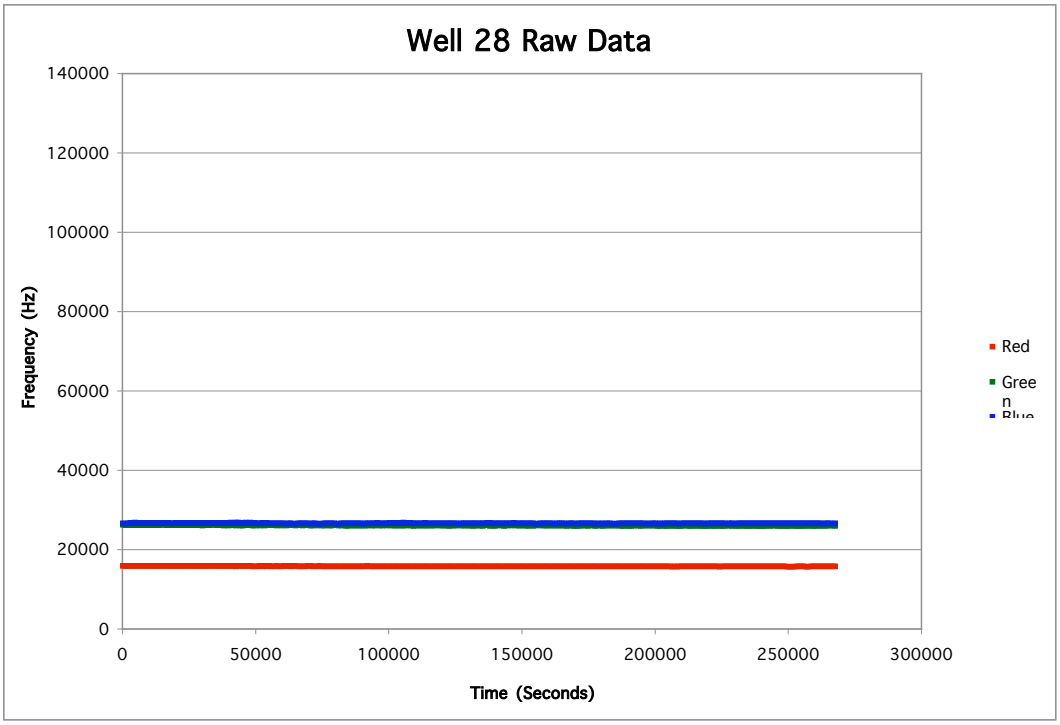
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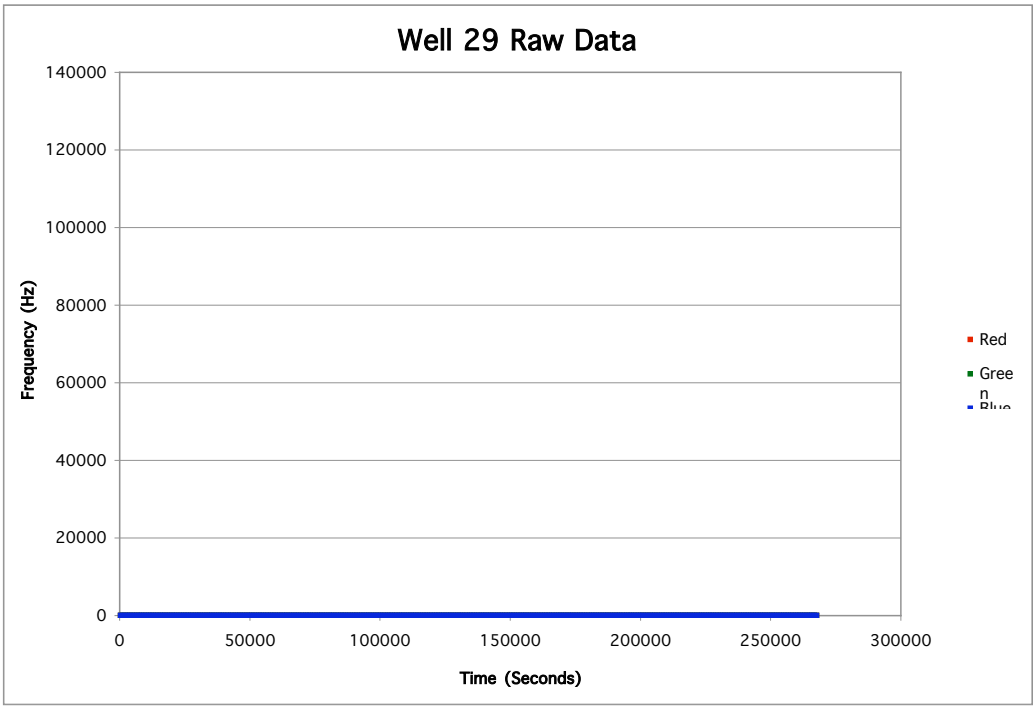
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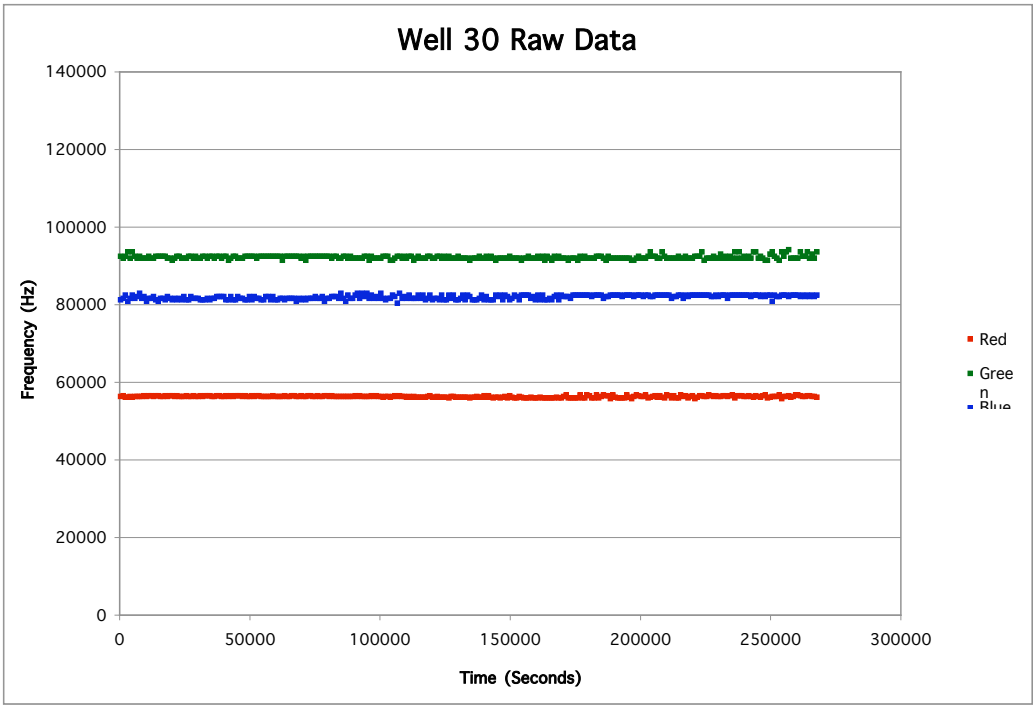
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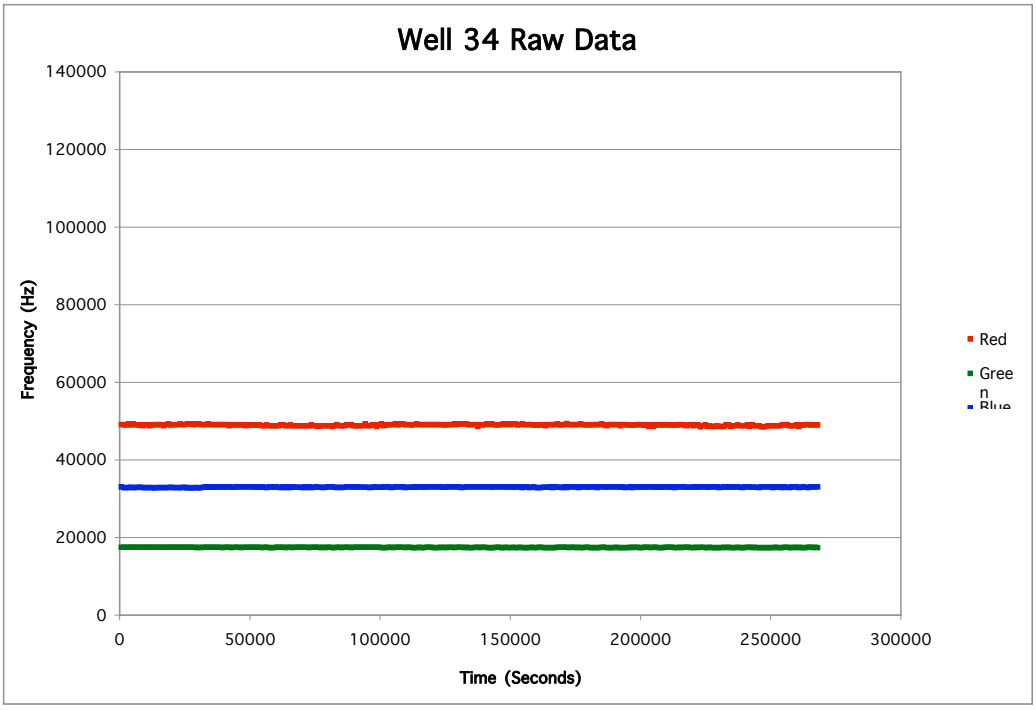
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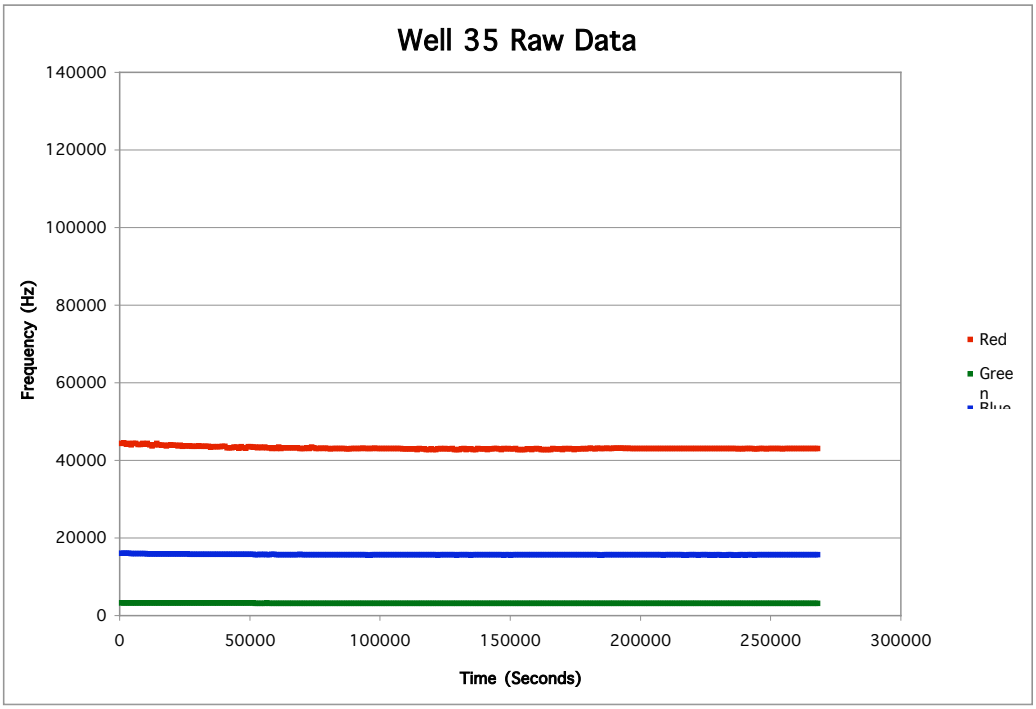
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